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Accumulation of Spliced Avian Retrovirus mRNA Is Inhibited in *S*-Adenosylmethionine-Depleted Chicken Embryo Fibroblasts

C. MARTIN STOLTZFUS* AND RICHARD W. DANE

Department of Microbiology, University of Iowa, Iowa City, Iowa 52242

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The synthesis and processing of B77 avian sarcoma virus RNA in infected chicken embryo fibroblasts was followed in the presence and absence of cycloleucine, a competitive inhibitor of the synthesis of *S*-adenosylmethionine and thus an inhibitor of RNA methylations. An increase in the steady-state levels of genome-length RNA and a decrease in the steady-state levels of subgenomic RNA molecules were obtained in the *S*-adenosylmethionine-depleted avian sarcoma virus-infected cells after 24 h of treatment with the inhibitor. The total number of virus-specific RNA molecules per cell, however, remained relatively constant under either condition. The production of newly synthesized virus-specific RNA in cycloleucine-treated and untreated cells infected with a transformation-defective strain of B77 avian sarcoma virus was followed as a function of [^{3}H]uridine labeling time. The accumulation of radioactive genome-length 8.4-kilobase (kb) RNA continued in cycloleucine-treated cells, and virus particle production proceeded at normal rates as previously shown by incorporation of labeled nucleoside precursors or amino acids. In contrast, newly synthesized 3.5-kb subgenomic mRNA, the putative mRNA for the envelope protein precursor, failed to accumulate in the treated cells. The extent of the inhibition in the appearance of the radioactive 3.5-kb RNA was correlated with the extent of the inhibition of viral genomic and cellular mRNA methylations and was a function of the cycloleucine concentration. Under conditions in which the accumulation of 3.5-kb envelope protein mRNA was blocked by the cycloleucine treatment, there were significant increases in the rate of synthesis of the polypeptide products of the genome-length RNA, the precursors to the non-glycosylated *gag* proteins (Pr76^{gag}), and the reverse transcriptase (Pr 180$^{gag\ pol}$) relative to the rate of synthesis of the envelope protein precursor (gPr 92^{env}). These results suggest that there is an *S*-adenosylmethionine requirement for the splicing, but not for the synthesis, packaging, or messenger function, of avian retrovirus genome-length RNA. Possible reasons for this requirement are discussed.

The 38S genomic RNA of avian sarcoma virions (ASV) contains four genes (45) with the following order, 5′ to 3′: *gag*, which codes for the non-glycosylated internal structural proteins p12, p15, p19, and p27; *pol*, which codes for the RNA-dependent DNA polymerase or reverse transcriptase; *env*, which codes for the glycosylated envelope proteins gp85 and gp35; and *src*, which codes for a nonstructural polypeptide of approximately 60,000 molecular weight (pp60^{src}) and whose presence is necessary for cell transformation (8). The 38S RNA has structural features in common with cellular mRNAs; i.e., the RNAs are polyadenylated [poly(A)$^+$] (26), possess a 5′-terminal blocked and methylated cap structure, m^{7}GpppG^m (20, 23), and contain 10 to 15 internal *N*-6-methyladenosine (m^{6}A) residues (5, 15, 20). The sequence specificity for the location of the m^{6}A residues is identical to that of host mRNA; all methylations appear in the sequences G-m^{6}A-C and A-m^{6}A-C (15). In addition, most if not all of the m^{6}A residues are located in the 3′ half of the ASV genome RNA (5). The viral 38S RNA can be translated in cell-free systems, where the major product is the 76,000-dalton *gag* precursor polypeptide (Pr76^{gag}) (34, 35, 46) and a minor product is the 180,000-dalton *gag pol* fusion protein (Pr180$^{gag\ pol}$) (32). It is not clear whether the latter polypeptide is formed by a translational read-through suppression mechanism or by the translation of an additional species of 38S RNA in which the termination codon of Pr76^{gag} has been deleted by a splicing mechanism (6). Intact 38S RNA does not appear to serve as mRNA for the *env* and *src* proteins. However, the *src* and

env proteins have been synthesized by the translation of subgenomic-size RNA that is present in purified virions (36, 37). It has not yet been established whether these translational activities are due to a small amount of packaged mRNA or to RNA fragments that result from endonucleolytic cleavage of 38S RNA and consequent exposure of cryptic sites for polypeptide initiation.

In ASV-infected cells at least three different virus-specific RNA size classes are present: 38S, 28S, and 21S (21, 47). It is thought that the 28S and 21S subgenomic RNAs are generated by means of a splicing process from the 38S genome RNA, which in turn is synthesized from an integrated DNA proviral template. The 38S genome RNA is presumed to be unspliced. The evidence for this proposal is as follows. Each of the smaller RNAs contains at least a 104-base 5'-leader sequence that is identical to the sequence at the 5' end of the genome RNA (13, 29, 40). The bodies of the 28S and 21S mRNAs, however, are derived from the 3' half and 3' third of the genome, respectively (21, 47), and contain *env* and *src* sequences. These RNAs presumably serve as messengers for the synthesis of the *env* and *src* proteins. It has also been shown that *env* mRNA is present when 35S genomic RNA from nontransforming avian leukosis virions is microinjected into the nuclei of cells infected with an *env* deletion mutant of Rous sarcoma virus. Since the same RNA species is inactive when microinjected into the cytoplasm of these cells, it suggests that the 35S RNA is processed in the cell nuclei to generate functional *env* mRNA molecules (39).

If the 28S and 21S mRNAs are indeed derived from 38S RNA, as appears to be the case, it is not clear what factors regulate the extent of the splicing process since, in contrast to most cellular RNA precursors, only a fraction of 38S RNA transcripts are converted to the smaller RNAs. It is possible that subtle differences exist in the structures of those 38S RNA molecules that are destined to be spliced to the various subgenomic mRNAs and those destined to serve as genomic RNA, *gag* mRNA, and *gag pol* mRNA. An intriguing possibility for directing RNA molecules with the same primary structure into different functional pools would be for these 38S molecules to be methylated in different ways. Such differences in RNA methylation may be recognized by viral or cellular proteins or both which are involved in the RNA splicing or in the transport of viral RNA. There are a number of proteins which demonstrate such specificity; bacterial restriction enzymes are examples (2, 38).

In this paper we report the effects of an in vivo inhibitor of methylation, cycloleucine (1-aminocyclopentane-1-carboxylic acid), on the levels of the presumptive spliced ASV mRNAs. Cycloleucine is a competitive inhibitor in vitro of *S*-adenosylmethionine (AdoMet) synthetase, and the cycloleucine concentration required to inhibit the reaction by twofold is in the range of 2 to 6 mM (14, 28). In vivo, in the presence of the inhibitor, the intracellular concentration of AdoMet is decreased (10). It has previously been shown that in the presence of 40 mM cycloleucine both 5'-terminal 2'-*O*-methyl ribose and internal m^6A methylations of mRNA from ASV-infected chicken embryo fibroblasts and ASV genomic RNA are greatly inhibited. Little or no inhibition of the 5'-terminal m^7G methylations occurs under these conditions (16, 17). In spite of this inhibition of methylation, cellular mRNA is synthesized, transported from the nucleus to the cytoplasm, and associated with polyribosomes (17). Furthermore, the production of ASV virions containing undermethylated 38S RNA continues at near-normal rates for at least 24 h after the addition of the inhibitor (16). It also appeared from our preliminary results that the gross size distribution of the total poly(A)$^+$ RNA from infected cells was not significantly altered in the presence of the inhibitor (17). However, because a large number of different cellular mRNAs were present in the RNA population, we could not determine the possible effects of undermethylation on the splicing of particular RNA transcripts. In this paper we report the effects of the AdoMet depletion on the production of spliced and unspliced ASV RNAs. We show that there is a striking difference in the size distribution of the virus-specific RNA when compared with RNA from the untreated control cells. In addition, newly synthesized subgenomic *env* mRNA does not accumulate in cycloleucine-treated infected cells, whereas the genome-length RNA accumulates in larger than normal amounts. Under conditions in which the formation of the *env* mRNA is blocked by the cycloleucine treatment, we show that there is a significant increase in the rate of synthesis of $Pr76^{gag}$ and a decrease in the rate of synthesis of the *env* protein precursor $gPr92^{env}$. This result is consistent with the observed effects on the levels of the viral mRNA species.

MATERIALS AND METHODS

Cells and viruses. Chicken embryo fibroblasts (virus negative, chick helper factor negative, avian leukosis virus group-specific antigen negative) from SPAFAS (Norwich, Conn.) were infected with B77 ASV or a transformation-defective strain of B77 ASV (td ASV) according to previously described techniques (42). Stocks of both viruses were generously provided by Peter K. Vogt, University of Southern California. The infected cells were passaged one or two times before the experiments described below were done.

Radioactive labeling and isolation of cellular and viral RNA. Cultures of chicken embryo fibroblasts infected with ASV or td ASV were treated for 12 to 14 h with Earle minimal essential medium containing 10 μM methionine and various concentrations of cycloleucine as described above (16). The media were then removed and replaced with appropriate media containing 50 μCi of [^{3}H]uridine (40 to 60 Ci/mmol; Amersham Corp.) or [^{3}H]adenosine (35 to 50 μCi/mmol; ICN) per ml. Incubations were stopped by removal of the media, and the cells were washed with ice-cold TBS (TBS = 140 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 5.5 mM glucose, 25 mM Tris, pH 7.2). Two methods were used to isolate whole-cell poly(A)$^+$ RNA. The first method (method 1) used the procedure described previously (40). In brief, cells were disrupted in 0.5% sodium dodecyl sulfate (SDS)–50 U of sodium heparin per ml–250 μg of proteinase K (EM Laboratories) per ml. After incubation of the disrupted cell preparation for 30 min at 37°C, a phenol-$CHCl_3$ extraction was carried out and the RNA was precipitated with 2 volumes of ethanol. The RNA was further purified by RNase-free DNase digestion, precipitation in 1.5 M LiCl solution, and oligodeoxythymidylate-cellulose chromatography. Isolation of RNA by the second method (method 2) was carried out essentially according to the procedure of Strohman et al. (43). In brief, the cells were harvested by the addition of a 19:1 (vol/vol) mixture of 8 M guanidine hydrochloride–2 M potassium acetate, pH 5.2. The RNA was isolated by a high-salt precipitation followed by oligodeoxythymidylate-cellulose chromatography.

Preparation of ^{32}P-labeled hybridization probes and hybridization conditions. DNA-RNA hybridization and the assay of DNA-RNA hybrids by S1 nuclease digestion were carried out as described previously (40). The preparation of DNA complementary to the 5′-terminal 100 bases and the 3′-terminal poly(A) sequences have also been described previously (19, 40, 44).

Isolation of radioactive virus-specific RNA. Virus-specific RNA was isolated by hybridization to cellulose to which *Eco*RI-cleaved pSal101 DNA, a recombinant plasmid containing the entire Prague A Rous sarcoma virus genome cloned at the *Sal*I site of pBR322, had been covalently linked. A stock of this plasmid was generously provided by J. Thomas Parson, University of Virginia. The procedure for the covalent linkage was essentially carried out according to the methods of Noyes and Stark (31). The hybridizations were carried out in 80% formamide–0.4 M NaCl–0.1 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), pH 6.5–0.005 M sodium EDTA (hybridization buffer) for 4 h at 50°C in a volume of 250 μl. The amount of DNA-cellulose used was at least a 10-fold excess with respect to the amount of viral RNA in the sample. The samples included radioactive poly(A)$^+$ RNA from infected cells, 60 μg of total RNA from a quail tumor cell line (QT-6) described by Moscovici et al. (30), 50 μl of yeast tRNA, and 10 μg of poly(A) (P-L Biochemicals). After the 4-h incubation, the cellulose was pelleted in an Eppendorf microfuge and the supernatants were removed. The cellulose was then washed according to the following protocol: two times with 500 μl of 2× SSC (SSC = 0.15 NaCl plus 0.015 M sodium citrate) at room temperature, four times with 500 μl of hybridization buffer at 55°C. The virus-specific RNA was then eluted with two washes of 250 μl of 99% formamide–0.01 M PIPES (pH 6.5) at 60°C, 2 ml of distilled water was added to dilute the formamide, and the solution was brought to a final concentration of 0.15 M sodium acetate. The RNA was precipitated at −20°C with 2.5 volumes of 95% ethanol. To improve the specificity of the isolation, the eluted RNA samples were subjected to a second cycle of hybridization. The amount of radioactive RNA hybridized in the second cycle was routinely 50 to 70% of the radioactivity which was bound in the first cycle. The RNA was precipitated in the presence of 15 μg of yeast tRNA as carrier. Viral RNA isolated in this manner was intact and could be translated in rabbit reticulocyte lysates to yield authentic virus-specific polypeptides (T. Ficht and C. M. Stoltzfus, manuscript in preparation).

Agarose gel electrophoresis of RNA. Electrophoresis of RNA in 1% agarose–10 mM methyl mercury hydroxide (Alpha Division, Ventron Chemical) was carried out essentially according to the procedures of Bailey and Davidson (4). After the electrophoresis was complete, the gel was dehydrated for 30 min with a solution of 95% ethanol–0.5% mercaptoethanol. This procedure was repeated and was followed by immersion of the dehydrated gel in 10% (wt/vol) 2,5-diphenyloxazole in acetone for 2 h at 37°C. The gel was then immersed in distilled water overnight to precipitate 2,5-diphenyloxazole, dried with heat under vacuum onto 3 MM Whatman paper, and exposed to Kodak Blue Brand film at −70°C in the presence of Cronex Lightning Plus intensifying screens.

Preparation of [^{3}H]leucine-labeled cytoplasmic extracts. Infected chicken embryo fibroblasts in 100-mm plastic petri dishes were treated with minimal essential medium containing 10 μM methionine and various concentrations of cycloleucine for 14 h. The media were then changed to appropriate media lacking leucine (5 ml per dish). After a 1-h incubation, 200 μCi of [^{3}H]leucine (40 Ci/mmol; ICN) was added to each dish and the cells were labeled for 1 h. The cells were washed once with TBS at 4°C, scraped into a solution (1 ml per 100-mm dish) containing 10 mM Tris-hydrochloride (pH 7.4), 1 mM NaCl, 1.5 mM $MgCl_2$, and 0.2 trypsin inhibitor units of Aprotinin (Sigma Chemical Co.) per ml, and swelled on ice for 10 min. The cell suspensions were then brought to a final concentration of 1% (vol/vol) Nonidet P-40, homogenized with five strokes of a Dounce homogenizer, and centrifuged for 30 min at 10,000 × *g* to remove nuclei and cellular debris. The supernatant was removed and stored in 500-μl aliquots at −70°C.

Immunoprecipitation of virus-specific polypeptides. The immunoprecipitation of immune complexes by Formalin-fixed *Staphylococcus aureus* Cowan I strain (SAC) by the methods of Kessler (24) was carried out according to previously described techniques (41). Appropriate aliquots of [^{3}H]leucine-labeled cell extracts containing a total of 6×10^5 cpm of protein were treated with 15 μl of control goat antiserum and 200 μl of a 10% (vol/vol) suspension of SAC for 30 min. The SAC was then pelleted by centrifugation and the supernatants were removed. To the supernatants an appropriate volume of antisera was added (usually 10 to 20 μl). After 30 min, 200 μl of a 10% suspension of SAC was added. After 10 min the SAC was pelleted, washed one time with 200 μl of a solution containing 150 mM NaCl, 5 mM EDTA, 50 mM Tris (pH 7.4),

0.5% (vol/vol) Nonidet P-40, 1% (wt/vol) bovine serum albumin, and 0.02% (wt/vol) sodium azide, and washed twice in 200 μl of the same solution lacking bovine serum albumin. The samples were then suspended in 50 μl of 6 M urea–4% (wt/vol) SDS and boiled for 3 min, and the SAC was pelleted by centrifugation. To the supernatants was added 5 μl of β-mercaptoethanol and 5 μl of a solution containing 1% (wt/vol) bromophenol blue in 80% glycerol. The samples were then loaded onto an SDS-polyacrylamide gel.

Gel electrophoresis of proteins. Discontinuous SDS-10% polyacrylamide slab gels were prepared essentially by the method of Laemmli (25). The samples were electrophoresed at 160 V until the tracking dye reached the bottom edge of the gel (approximately 10 cm). Radioactive labeled proteins were located by fluorography essentially as described by Bonner and Laskey (7).

RESULTS

Steady-state size distribution of ASV RNA in cycloleucine-treated and control infected cells. Poly(A)$^+$ RNA preparations from B77 ASV-infected cells which had been treated for 24 h with 40 mM cycloleucine in cell culture medium containing 10 μM methionine or from parallel cultures of infected cells treated with medium containing 10 μM methionine (low-methionine control) were sedimented on 5 to 30% glycerol gradients. In this experiment the cells were labeled with [^{3}H]uridine for 6 h before isolation of the RNA. The profiles of the two labeled RNA preparations were similar; both exhibited broad size distributions with peaks at about 16S (Fig. 1). There was, however, a significant increase in the amount of radioactive RNA in the region between 28S and 42S in the cycloleucine-treated relative to the low-methionine control cells.

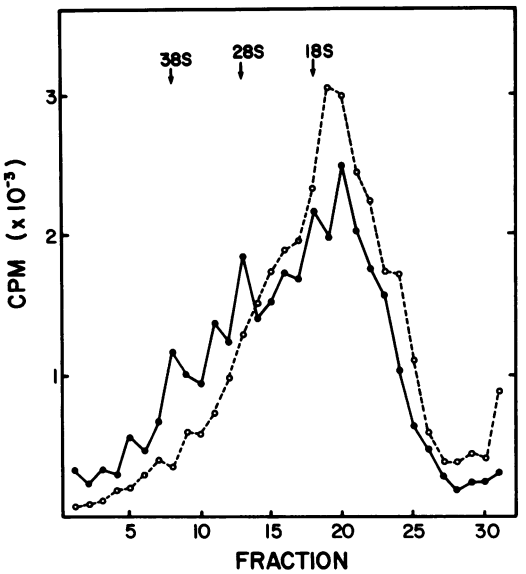

FIG. 1. Glycerol gradient sedimentation of [^{3}H]uridine-labeled total poly(A)$^+$ RNA from B77 ASV-infected chicken embryo fibroblasts. Poly(A)$^+$ RNA from B77 ASV-infected chicken embryo fibroblasts treated for 24 h in the presence (—) and absence (----) of 40 mM cycloleucine was isolated by method 1 (see text). The RNA samples in volumes of 0.2 ml were applied to 5 to 30% glycerol gradients and sedimented for 16 h at 25,000 rpm and 4°C in a Beckman SW41 rotor. Samples of 0.4 ml were collected, and aliquots of 5 μl were counted for radioactivity.

An aliquot from each gradient fraction was hybridized either to a ^{32}P-labeled cDNA probe that is complementary to the 5′-terminal 100 bases of the 38S genomic RNA (5′-cDNA$_{100}$) or to the sequences immediately adjacent to the 3′-terminal poly(A) sequence of the 38S RNA (3′-cDNA). The subgenomic mRNAs each contain a 5′-leader sequence identical to the sequence at the 5′ terminus of the genomic RNA (13, 29, 40). The 5′-cDNA probe therefore detects the presence and amounts of the putative spliced mRNAs. In contrast, the hybridization of RNA to 3′-cDNA is an assay for the bodies of the mRNAs and detects both spliced and unspliced subgenomic RNA as well as degraded 38S RNA. It was clear from the magnitudes of the percent hybridization values when either 3′- or 5′-cDNA probes were used that in the cycloleucine-treated cells, compared with the low-methionine control cells, the amounts of the presumptive spliced 28S and 21S subgenomic mRNAs were considerably reduced relative to the amounts of 38S RNA (Fig. 2). Additional virus-specific RNA molecules sedimenting at a velocity of <18S were also present in the control infected cells, and their concentration was also reduced in the cycloleucine-treated cells. Such RNA species have previously been reported by other investigators, and their detailed structure and significance remain unknown (9).

Quantitation of the amounts of ASV RNA by hybridization kinetics. The previous results suggested that in cycloleucine-treated ASV-infected cells the amounts of 38S viral RNA were increased relative to the amounts of 28S and 21S subgenomic mRNAs. Since the assay for 5′ sequences is specific for the presumptive spliced 28S and 21S mRNAs, it was possible that subgenomic RNAs lacking the 5′-leader sequence were formed in cycloleucine-treated cells. If this were the case, the relative concentration of the smaller poly(A)$^+$ RNA molecules containing sequences complementary to 3′-cDNA would exceed the concentration of smaller poly(A)$^+$ RNA molecules containing sequences complementary to 5′-cDNA.

To make this determination, we more carefully quantitated the concentrations of the 5′ and 3′

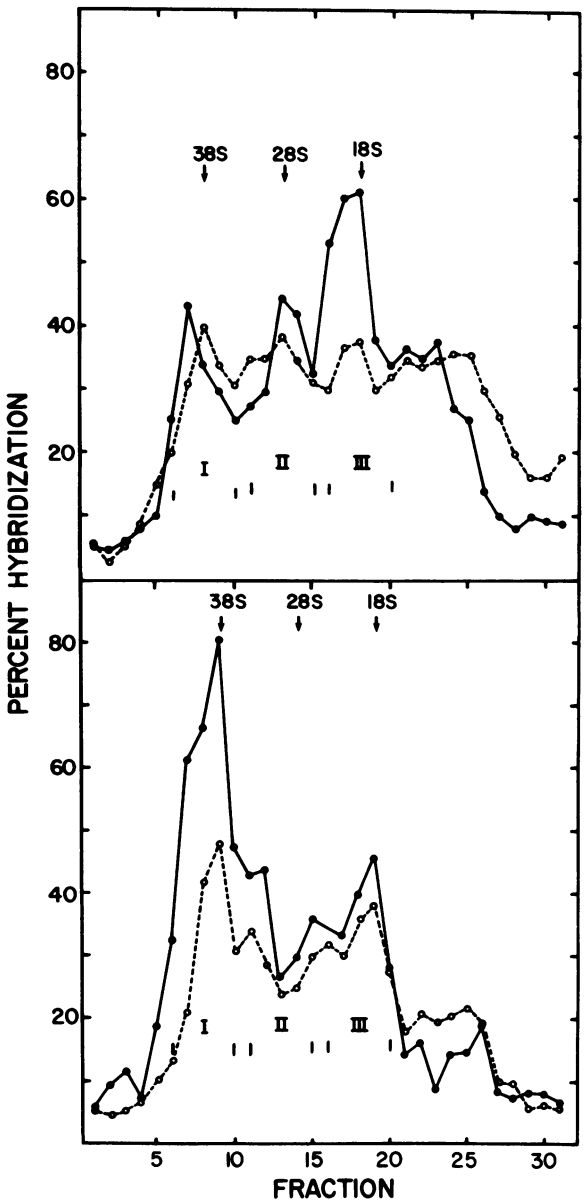

FIG. 2. Hybridization of fractions from glycerol gradients with 5'-cDNA and 3'-cDNA. Appropriate aliquots from each gradient fraction of poly(A)$^+$ RNA isolated from cycloleucine-treated (bottom) and control (top) cells (see Fig. 1) were hybridized to 5'-cDNA$_{100}$ (—) and 3'-cDNA (----). (Aliquots of 20 μl were used for 5'-cDNA$_{100}$ and 10 μl were used for the 3'-cDNA.)

sequences. The fractions containing three RNA size classes (Fig. 2) were pooled and subjected to a second cycle of oligodeoxythymidylate-cellulose binding and elution to select against possible degradation products containing 5' sequences. The concentration of sequences complementary to 5'- and 3'-cDNA were then determined by hybridization kinetics. These results indicated that, whereas the $C_rt_{1/2}$ values for the 34 to 42S RNA pools were similar, those for the 15 to 23S and 25 to 31S RNA pools were increased by a factor of two to three in the cycloleucine-treated cells (Fig. 3). This implied that the relative ratio of genome-length RNA to subgenomic RNA was increased in cycloleucine-treated cells and was in agreement with the results given in Fig. 2. From the $C_rt_{1/2}$ values, the fractions of virus-specific RNA in the three

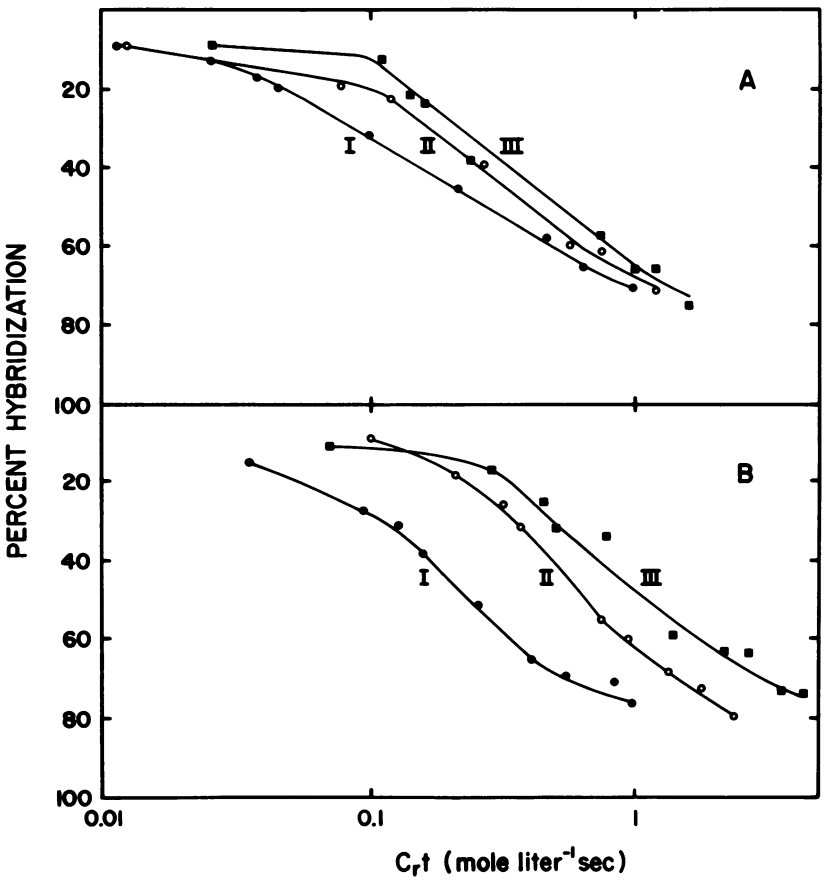

FIG. 3. Hybridization kinetics of various RNA size classes. Fractions from the glycerol gradients were combined into three pools as shown in Fig. 2. The RNA in each pool was subjected to oligodeoxythymidylate-cellulose chromatography. The three different poly(A)$^+$ RNA pools, which in each case represented at least 70% of the original RNA, were hybridized to both 5'-cDNA$_{100}$ and 3'-cDNA. The results are given for the hybridization of the RNA to the 5'-cDNA.

pools were determined (Table 1). The results indicated that in both the cycloleucine-treated and low-methionine control cells the concentrations of sequences complementary to the 5'-cDNA approximated the concentrations of sequences complementary to the 3'-cDNA in each RNA size class. It was concluded from these results that significant amounts of subgenomic RNA molecules lacking 5'-leader sequences do not accumulate in cycloleucine-treated cells. It was further concluded that the increase in the concentration of subgenomic RNA molecules in the low-methionine control cells relative to the cycloleucine-treated cells was not due to increased degradation of genome-length RNA.

Given the $C_rt_{1/2}$ values, the total amount of RNA in each pool, and the number of cells, the number of virus-specific molecules per cell in the RNA size classes was calculated. In the cycloleucine-treated cells there was a greater than twofold increase in the number of virus-specific molecules in the 34 to 42S size class and an approximately twofold reduction in the number of virus-specific molecules in the 25 to 31S and 15 to 23S size classes. The total number of virus-specific RNA molecules per cell appeared to remain relatively constant under both conditions (Table 1). The quantitative results given in Table 1 are thus consistent with the more qualitative results obtained in Fig. 2; there is a three- to fourfold increase in the relative ratio of 38S RNA to 28S and 21S RNA molecules under conditions in which RNA methylations are inhibited by the cycloleucine treatment. The differences in the relative amounts of 38S RNA and subgenomic mRNAs shown in Fig. 2 were reproducibly obtained in a number of independent experiments with both the B77 ASV and the Prague A strain of Rous sarcoma virus (data not shown).

TABLE 1. Number of molecules of virus-specific poly(A)$^+$ RNA in various size classes in cycloleucine-treated and control B77 ASV-infected cells

Condition	Pool	Total RNA (μg)	5′ Probe		3′ Probe		5′/3′ ratio	Molecules per cell[a]
			$C_rt_{1/2}$	Fraction of RNA[b]	$C_rt_{1/2}$	Fraction of RNA		
− Cycloleucine	34–42S	3.7	2.0×10^{-1}	0.15	9.0×10^{-2}	0.13	1.2	526
	25–31S	10.2	2.9×10^{-1}	0.051	9.9×10^{-2}	0.061	0.84	1,170
	15–23S	15.6	3.5×10^{-1}	0.028	1.5×10^{-1}	0.027	1.04	1,250
+ 40 mM cycloleucine	34–42S	7.2	1.9×10^{-1}	0.16	7.6×10^{-2}	0.16	1.0	1,433
	25–31S	12.4	5.0×10^{-1}	0.030	2.5×10^{-1}	0.024	1.3	700
	15–23S	16.8	1.0	0.010	3.4×10^{-1}	0.012	0.83	700

[a] The number of molecules per cell was estimated by the following formula: (micrograms of total RNA in pool/number of cells) × fraction of virus-specific RNA in pool × number of molecules per microgram of RNA. The preparations represented a total of 13 100-mm tissue culture dishes. Each dish contained 10^7 cells in the cycloleucine-treated and 1.2×10^7 cells in the low-methionine control samples. The RNA concentrations were determined by optical density measurements assuming 1 mg/ml is equivalent to 20 optical density units at 260 nm.

[b] The fraction of virus-specific RNA was determined by the following formula: [($C_rt_{1/2}$ for genome RNA/$C_rt_{1/2}$ for RNA pool)] × [(complexity of RNA in pool/complexity of genome RNA)]. The molecular complexities of the genome RNA and the RNA in pools I, II, and III were assumed to be 10, 10, 5, and 3 kb, respectively. The $C_rt_{1/2}$ values for the genome RNA were determined to be 3.0×10^{-2} and 1.2×10^{-2} for the 5′- and 3′-cDNA, respectively.

Accumulation kinetics of viral RNA in td B77 ASV-infected cells treated with cycloleucine. The previous results suggested that the formation of virus-specific subgenomic RNA is inhibited in the cycloleucine-treated cells. However, since the steady-state levels of viral RNA in infected cells are a composite of the rates of synthesis, turnover, and packaging into virions, the actual magnitude of the inhibition could not be determined. It was not clear, for instance, how much of the virus-specific RNA remaining in the cycloleucine-treated cells was formed before the addition of the inhibitor because the half-lives of the various viral RNA species were not known. Furthermore, the observed differences in the steady-state levels of the various RNA species were relatively small.

A method was therefore used in which only viral RNA synthesized after the addition of the methylation inhibitor was analyzed. To simplify the analysis of the RNA species, we studied the effects of cycloleucine on cells infected with a mutant of B77 ASV containing a deletion of the entire *src* gene (td B77). In these cells only two virus-specific RNA species appear to be present: 21S (3.5 kilobases [kb]) and 35S (8.4 kb), the putative spliced *env* mRNA and unspliced genome-length RNA, respectively (21, 47). The infected cells were treated for 12 h in either low-methionine medium containing 40 mM cycloleucine or low-methionine medium and then labeled with [^{3}H]uridine for various time intervals. The total poly(A)$^+$ RNA from cells was isolated, and the radioactive virus-specific RNA was selected by two cycles of hybridization to viral DNA-cellulose, as described in Materials and Methods. Approximately the same fraction of the cellular RNA from either the low-methionine control or the cycloleucine-treated cells hybridized to the viral DNA-cellulose at each labeling interval (Table 2). This result was consistent with the results presented in Table 1. It was also apparent from this analysis that the fraction of the total RNA represented by viral RNA increased as a function of the labeling time for at least 24 h. This result suggests that a longer time is required for the viral RNA to reach steady-state labeling conditions than for the bulk cellular poly(A)$^+$ RNA and therefore that the turnover rate of the viral RNA may be slower than for the bulk cellular mRNA.

The samples containing virus-specific RNA were then electrophoresed on 1% agarose gels containing 10 mM methyl mercury hydroxide.

TABLE 2. Percentage of total [^{3}H]uridine-labeled poly(A)$^+$ RNA hybridized to viral DNA-cellulose at various labeling intervals[a]

Labeling time (h)	Virus-specific RNA [% of total poly(A)$^+$]	
	10 μM Met MEM[b]	10 μM Met MEM + 40 mM cycloleucine
1	3.8	1.7
6	4.6	4.6
12	6.2	4.2
24	8.3	8.8
48	10.1	9.1

[a] Data are from experiments shown in Fig. 4 except for 12-h time point, which are data from the experiment shown in Fig. 6.

[b] Met MEM, Minimal essential medium containing 10 μM methionine.

The gel was prepared for fluorography and exposed to X-ray film as described above. In the cells incubated in the low-methionine control medium, radioactivity appeared first in the 8.4-kb RNA and little or no radioactivity was detectable in the spliced 3.5-kb band until the labeling was carried out for at least 6 h (Fig. 4). The amount of label in the 3.5-kb RNA band at the 24- and 48-h time points then increased relative to that in the 8.4-kb RNA band. In other experiments not shown we could not detect radioactivity in the 3.5-kb RNA band when the cells were labeled for intervals of 15 min to 1 h; all of the radioactivity was present in the 8.4-kb RNA band. These results suggest that a relatively small fraction of the 8.4-kb RNA is used as a precursor for the 3.5-kb RNA but that the 3.5-kb RNA turns over less rapidly and accumulates in the cells relative to the 8.4-kb RNA. This is reasonable since a substantial fraction of the 8.4-kb RNA is packaged into virions and exported from the cells. On the other hand, little or none of the 3.5-kb RNA is packaged (C. M. Stoltzfus, unpublished data). The slow accumulation of subgenomic B77 ASV RNA species relative to the genome-length RNA has been reported previously by other investigators (33).

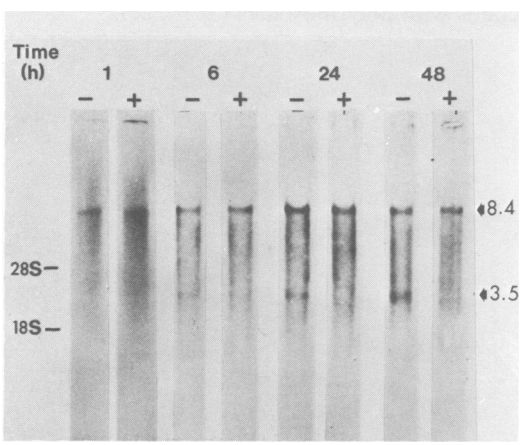

FIG. 4. Effect of cycloleucine treatment on appearance of newly synthesized viral RNA in td B77 ASV-infected cells. Infected cells were labeled in the appropriate medium with [^{3}H]uridine after a 12-h pretreatment with medium containing either 10 μM methionine (−) or 10 μM methionine and 40 mM cycloleucine (+). The total RNA was isolated by method 2 (see text), and the hybridization to viral DNA-cellulose was carried out as described in the text. The samples (approximately 5×10^4 cpm of the 6-, 24-, and 48-h samples and 1.5×10^4 cpm of the 1-h samples) were applied to horizontal 1% agarose gels containing 10 mM methyl mercury hydroxide and electrophoresed for 4 h at 95 V. The specific activities of the RNA preparation used in this experiment and in the experiments of Fig. 5 and 6 were all approximately 5×10^5 cpm/μg of RNA. The bands were detected by fluorography as described in the text. The exposure time for the 6-, 24-, and 48-h samples was 4 days, and that for the 1-h sample was 2 weeks.

In the cells treated with 40 mM cycloleucine there was a nearly complete inhibition in the accumulation of the 3.5-kb RNA band, whereas the appearance of the 8.4-kb RNA species was unaffected (Fig. 4). The latter observation is consistent with the fact that the synthesis of B77 ASV particles labeled with nucleosides or amino acid precursors proceeds at nearly normal rates in cycloleucine-treated cells (16). If cycloleucine blocks the synthesis of AdoMet in the cell by competitively inhibiting the methionine-ATP transferase activity (28), it should be possible to at least partially negate the effects of the inhibitor by adding increased amounts of exogenous methionine to the medium together with cycloleucine. Infected cells were labeled for 6 h in the presence and absence of 40 mM cycloleucine and containing either 10 μM methionine as in the previous experiments or 100 μM methionine. The virus-specific RNA was then isolated by DNA-cellulose hybridization and examined by gel electrophoresis. Note that, as previously shown in Fig. 4, a band of radioactivity corresponding to the 3.5-kb RNA did not appear in the cells treated with cycloleucine and 10 μM methionine, whereas the band was clearly present in the cells treated with 10 μM methionine alone (Fig. 5). However, when the methionine concentration was increased to 100 μM, the 3.5-kb RNA band was present in both the cycloleucine-treated and the control cells. The ability to at least partially reverse the inhibition of the accumulation of 3.5-kb RNA by increasing the methionine concentration suggested that the effect of cycloleucine was due to a decrease in AdoMet levels in the cells rather than to an indirect effect of cycloleucine on the cells. However, it cannot be entirely excluded from this data that methionine is inhibiting the uptake of cycloleucine and by this means reversing the effect.

Effect of various cycloleucine concentrations on RNA methylations and on amounts of subgenomic RNA. To correlate different levels of mRNA methylation with the effects on the processing of viral RNA, we treated td B77 ASV-infected cells with various concentrations of cycloleucine, labeled the RNA produced under these conditions with [^{3}H]adenosine or [^{3}H]uridine for 12 h, and then determined the size distribution of equal amounts of hybrid-selected radioactive viral RNA by gel electrophoresis. An increase in the cycloleucine concentration resulted in a decrease in the proportion of the total label in the 3.5-kb RNA band compared with the 8.4-kb band (Fig. 6). This effect was most pronounced

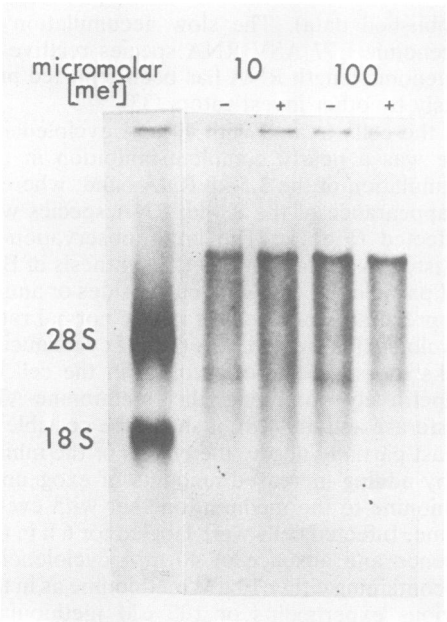

FIG. 5. Effect of increased methionine concentration on appearance of newly synthesized viral RNA in td B77-infected cells in the presence and absence of cycloleucine. Infected cells were labeled in appropriate media with [^{3}H]uridine after a 12-h pretreatment with medium containing either 10 μM methionine or 100 μM methionine in the presence (+) and absence (−) of 40 mM cycloleucine. RNA isolation, electrophoresis, and fluorography were carried out as described in the legend to Fig. 4. Approximately 4×10^4 cpm was applied to each slot, and the exposure time was 6 days.

when the cycloleucine concentration was greater than 10 mM. To test the levels of mRNA methylation at each of the cycloleucine concentrations, we analyzed RNase T_2 hydrolysates of [^{3}H]adenosine-labeled RNA for the ratios of radioactivity in m^6A residues to that in adenosine residues. The internal methylations of both virion RNA and cellular mRNA appear to be inhibited by approximately the same extent as a function of increasing cycloleucine concentration (Fig. 7). We concluded from these results that the effects of cycloleucine on the processing of viral RNA were dose dependent and that the concentration of cycloleucine required to inhibit the accumulation of 3.5-kb RNA (10 and 40 mM) also inhibited the internal methylations of the viral and cellular RNA by >80%. We have previously shown that, at a concentration of 40 mM cycloleucine, the 2′-*O*-methyl ribose cap methylations of viral and cellular mRNAs are also inhibited by >90% (16, 17).

Effects of cycloleucine on synthesis of virus-specific polypeptides. As a consequence of the inhibition of *env* mRNA production and the accumulation of presumably unspliced genome-length RNA, an increase in the amounts of the gene products synthesized from the genome-length RNA relative to the amounts of gene products synthesized from *env* mRNA might be expected in cycloleucine-treated cells. To test for this possibility, td B77 ASV-infected cells were treated for 15 h with media containing various concentrations of cycloleucine. The cells were then labeled with [^{3}H]leucine for 1 h, and cell extracts were prepared. When equal amounts of radioactive protein from cells treated with media containing various concentrations of cycloleucine were electrophoresed on SDS-polyacrylamide gels, there was little if any difference in the resulting polypeptide profiles (Fig. 8). These patterns, however, are complex and small differences would probably not be detected at this relatively crude level of resolution. The results do suggest that the synthesis of numerous cellular polypeptides continues in the cycloleucine-treated cells. This is in agreement with previous studies which have indicated that undermethylated cellular poly(A)$^+$ RNA associates with polyribosomes (3, 17, 22).

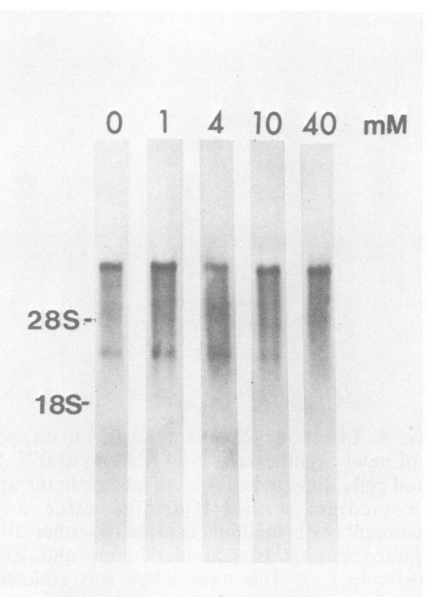

FIG. 6. Effect of various concentrations of cycloleucine on accumulation of genomic and subgenomic RNA in td B77 ASV-infected cells. Infected cells were treated with media containing various concentrations of cycloleucine as indicated in the presence of 10 μM methionine for 12 h. The cells were then labeled with [^{3}H]uridine for 12 h. RNA isolation, electrophoresis, and fluorography were carried out as described in the legend to Fig. 4, and the exposure time for fluorography was 4 days. Approximately 5×10^4 cpm was applied to each slot.

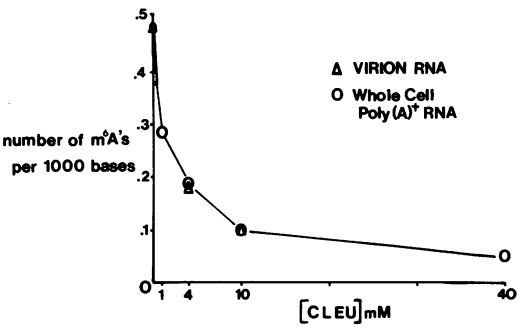

FIG. 7. Effect of various concentrations of cycloleucine (CLEU) on internal methylations of viral genomic RNA and cellular mRNA. [^{3}H]adenosine-labeled poly(A)$^+$ RNA samples from td B77 ASV-infected cells which were treated with media containing various concentrations of cycloleucine or [^{3}H]adenosine-labeled 35S RNA samples isolated from td B77 ASV virions produced by infected cells treated with various concentrations of cycloleucine were digested with 50 U of T2 RNase per ml for 3 h at 37°C. The amounts of radioactivity in 5'-AMP and 5'-m^{6}AMP were determined by paper chromatography as described previously (15). The mole percent adenosine residues in the RNA samples was assumed to be 25% for the purpose of the calculations shown.

Virus-specific polypeptides in equal amounts of radioactive protein from the labeled cell extracts were immunoprecipitated with excess antiserum directed against disrupted whole avian myeloblastosis virus particles. This antiserum immunoprecipitates both *gag* and *env* polypeptides from B77 ASV virions and therefore is capable of immunoprecipitating both the *gag* protein precursor Pr76^{gag} and the *env* protein precursor gPr92^{env} (data not shown). Because of the activity against *gag* proteins, the reverse transcriptase precursor Pr180$^{gag\ pol}$ was also immunoprecipitated by the antiserum. It was found that in the immunoprecipitates from extracts of cells treated with 40 mM cycloleucine (Fig. 9A, lane f) there was a dramatic increase relative to the low-methionine control (lane b) in the amounts of labeled Pr76^{gag} and the *gag* cleavage products Pr66^{gag}, Pr60^{gag}, and p27. This is accompanied by a relative decrease in the amount of labeled gPr92^{env}. At cycloleucine concentrations of 1 (lane c) and 4 (lane d) mM there appeared to be little effect on the relative amounts of various virus-specific polypeptides. At a concentration of 10 mM cycloleucine (lane e) there appeared to be some decrease in the amount of gPr92^{env} and an increase in the amount of p27 relative to the low-methionine control. The amount of pr76^{gag}, however, did not appear to increase in concert with the amount of p27, one of the final cleavage products of pr76^{gag}. These results were confirmed by densitometric analysis of the fluorogram (data not shown).

In addition to the dramatic apparent increase in the rate of synthesis of *gag* polypeptides in cells treated with 40 mM cycloleucine, there was a corresponding increase in the rate of synthesis of Pr180$^{gag\ pol}$. The identity of this polypeptide was confirmed by demonstrating that it could be immunoprecipitated with an antiserum directed against reverse transcriptase (Fig. 9B, lane f). Under the exposure conditions for fluorography used in the experiment shown, detectable levels of Pr180$^{gag\ pol}$ were present in cells treated with 10 mM cycloleucine (lane e) but not in cells treated with lower cycloleucine concentrations (lanes b, c, and d).

Results similar to those given in Fig. 9 were ob-

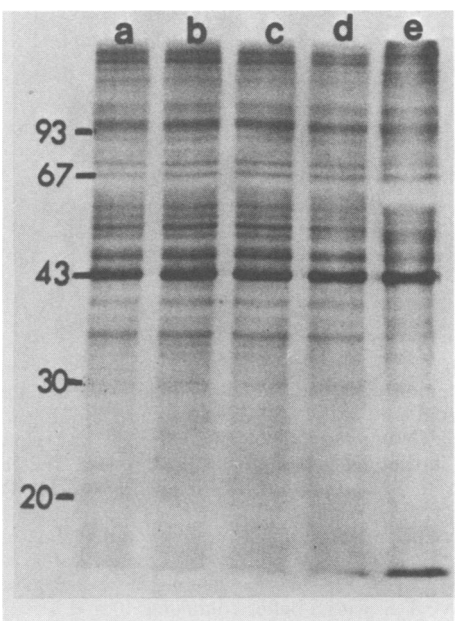

FIG. 8. Effects of various concentrations of cycloleucine on synthesis of total cellular polypeptides. Cells infected with td B77 ASV were treated for 15 h with media containing various concentrations of cycloleucine. The cells were labeled with [^{3}H]leucine and extracts were prepared according to methods described in the text. Equal amounts (2×10^4 cpm) of radioactively labeled extracts were disrupted in 20 μl of a solution containing 6 M urea–4% SDS–5% β-mercaptoethanol, boiled for 1 min, and applied to SDS-polyacrylamide gels as described in the text: extracts from cells treated with low methionine (lane a) or 1 mM (lane b), 4 mM (lane c), 10 mM (lane d), or 40 mM cycloleucine (lane e). The positions of the molecular weight marker proteins (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin) are given.

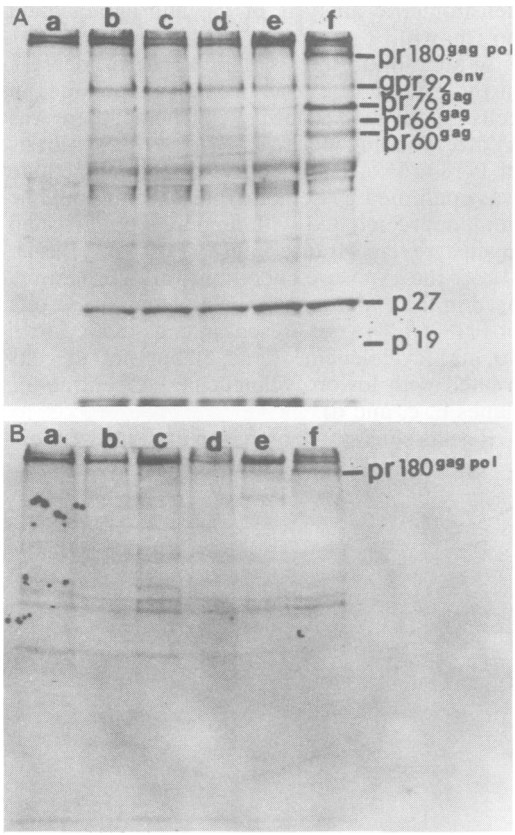

FIG. 9. Effects of various concentrations of cycloleucine on virus-specific polypeptide synthesis. Cells infected with td B77 ASV were treated for 15 h with media containing various concentrations of cycloleucine in the presence of 10 μM methionine. The cells were then labeled with [^{3}H]leucine, extracts were prepared, and immunoprecipitations were carried out according to methods described in the text. For each sample, equal amounts (6×10^5 cpm) of radioactive protein were used, and the volumes were each adjusted to 0.4 ml with a buffer containing 10 mM Tris (pH 7.4)–1 mM NaCl–1.5 mM $MgCl_2$. Polyacrylamide gel electrophoresis and fluorography were carried out according to methods described in the text. (A) Immunoprecipitation with anti-avian myeloblastosis virus (AMV) serum: (lane a) low-methionine control extract immunoprecipitated with 15 μl of control goat antiserum; extracts from cells with low methionine (lane b) and 1 mM (lane c), 4 mM (lane d), 10 mM (lane e), or 40 mM cycloleucine (lane f) immunoprecipitated with 15 μl of goat antiserum directed against detergent-disrupted AMV. (B) Immunoprecipitation with anti-reverse transcriptase serum: (lane a) low-methionine control extract immunoprecipitated with 20 μl of control rabbit serum; extracts from cells with low methionine (lane b) and 1 mM (lane c), 4 mM (lane d), 10 mM (lane e), or 40 mM cycloleucine (lane f) immunoprecipitated with 20 μl of rabbit antiserum directed against purified reverse transcriptase.

tained from immunoprecipitates prepared from cells infected with nondefective ASV (data not shown). We concluded from these results and from those given in Fig. 9 that the relative rates of synthesis of the polypeptide products of the genome-length RNA, Pr76^{gag} and Pr180$^{gag\ pol}$, were increased, whereas the rate of synthesis of gPr92^{env} was decreased in cells treated with medium containing cycloleucine concentrations of >10 mM. These results were consistent with the results obtained above on the changes in the relative mRNA levels.

DISCUSSION

We have shown that the inhibition of AdoMet-dependent methylations by cycloleucine in avian retrovirus-infected chicken embryo fibroblasts results in an inhibition in the accumulation of spliced viral mRNA. The unspliced viral genome-length RNA continues to accumulate under these conditions as evidenced by (i) the production of virus particles containing undermethylated genomic RNA (16), (ii) the presence of newly synthesized 8.4-kb genome RNA in td B77 ASV-infected cells (Fig. 4), and (iii) the continued synthesis of viral polypeptides Pr76^{gag} and Pr180$^{gag\ pol}$ in infected cells which have been treated for 15 h with cycloleucine (Fig. 9). We cannot yet conclusively determine from our data whether the splicing of the genome-length RNA is inhibited or whether the spliced mRNA is formed and is rapidly degraded. We favor the former alternative for the following reasons. First, the total number of virus-specific RNA molecules per cell calculated by hybridization kinetics measurements remains relatively constant after a 24-h treatment with 40 mM cycloleucine in spite of the significant difference in the size distribution (Table 1). This result suggests that the viral genome-length RNA molecules accumulate at the expense of the spliced subgenomic RNA molecules which we have shown are not formed under these conditions. Second, in the presence of cycloleucine, the rates of synthesis of the *gag* polypeptides and reverse transcriptase precursor Pr180$^{gag\ pol}$ are increased significantly, whereas the rate of synthesis of the *env* precursor gPr92^{env} is decreased (Fig. 9). The cycloleucine dose response for these effects appears to be similar to that observed for the inhibition of *env* mRNA accumulation (cf. Fig. 6 and 9), suggesting that the effects on the synthesis of viral proteins are related to the effects on the accumulation of *env* mRNA. The simplest explanation for these observations is that the splicing of the genome-length RNA, which is the message for Pr76^{gag} and Pr180$^{gag\ pol}$, is inhibited and therefore that the genome-length RNA accumulates

in larger than normal amounts. The formation of the *env* mRNA, on the other hand, is inhibited and its message activity in the infected cells decreases. However, the observed increase in the relative amounts of $Pr76^{gag}$ in cells treated with 40 mM cycloleucine appears to be somewhat greater either than the observed increases in the amounts of genome-length RNA or in the amounts of p27. This results suggests that there may be additional effects of the cycloleucine treatment on the rate of cleavage of $Pr76^{gag}$. This possibility is currently under investigation.

The mechanism by which $Pr180^{gag\ pol}$ is formed in infected cells is not yet clear. One hypothesis has been proposed which suggests that $Pr180^{gag\ pol}$ is translated from a full-length transcript in which the normal termination codon of $Pr76^{gag}$ is spliced out (6). If this is the case, it would appear that accumulation of this putative spliced mRNA, in contrast to the *env* mRNA, is not inhibited in AdoMet-depleted cells.

Because there are numerous AdoMet-requiring processes in the cell, it is difficult at this point to ascribe the effects of cycloleucine on the accumulation of spliced viral RNA to any one of these processes. An attractive hypothesis, however, which has already been suggested as a possibility by a number of other investigators (1, 11, 12), implicates internal m^6A RNA methylations in influencing the splicing of mRNA precursors. It has been shown that, in adenovirus-infected cells, the precursor RNA transcripts from the major late promoter are methylated internally soon after their synthesis and before their splicing. Furthermore, in contrast to nonmethylated RNA sequences of the precursors which are conserved in the cytoplasm as stable mRNA to the extent of only about 20%, the m^6A-containing sequences are completely or nearly completely conserved (12). Since the generation of late adenovirus mRNAs involves the use of a number of alternative splice sites, Chen-Kiang et al. (12) have suggested that m^6A may play a role in the selection of these sites. They have proposed two possible models: (i) the splicing enzymes begin at the 3′ end of a large poly(A)$^+$ RNA and search for an unmethylated splice site; or (ii) the search begins at the 5′ end and splicing occurs near the first methylation. If the splicing process requires an unmethylated splice site, an increase in the amount of spliced mRNA in cycloleucine-treated cells would be expected. On the other hand, if the splicing process involves a search for methylated sites, a decrease in the amount of spliced mRNA would be expected. Therefore, the results reported in this paper would be more consistent with the second model.

We do not yet know the effects of cycloleucine and the subsequent methylation inhibition on the splicing of specific host cell mRNA precursors. It appears from our results that, although the overall size distribution of the total poly(A)$^+$ RNA is similar in both treated and untreated cells, there is an increase in the amount of labeled RNA from the cycloleucine-treated cells in the 34 to 42S RNA size class (Fig. 1). At least part of this increase can be accounted for by the increase in the amount of viral RNA of this size. However, this cannot entirely explain the result since an increase in the fraction of virus-specific RNA was not observed in this pool (Table 1). These results imply that there is a corresponding increase in the amounts of cellular RNA of this size class in the cycloleucine-treated cells. Bachellerie et al. (3) have previously reported that there is an increase in the size distribution of 1-h pulse-labeled poly(A)$^+$ nuclear RNA in cycloleucine-treated Chinese hamster ovary cells. After a chase period of 2 h, however, the differences in size distribution were no longer apparent. This result suggested that methylated RNA precursors may undergo a slower processing in these cells. It was also determined from these studies that the average half-life of nuclear poly(A)$^+$ RNA formed in the presence of cycloleucine is more than twice the half-life for control nuclear poly(A)$^+$ RNA (3). These results suggested that the block of RNA methylation may decrease the rate but not the final extent of processing.

The metabolism of retrovirus RNA is unusual and differs from the fate of most cellular mRNA precursors in that the primary viral RNA transcripts can either be spliced or remain unspliced. Indeed, a large percentage of the transcripts in td B77- and nondefective B77 ASV-infected cells appear not to be spliced and are packaged into virions (Stoltzfus and Ficht, unpublished data). Therefore, as proposed by other investigators, there may be active participation of viral RNA binding proteins such as the *gag* protein p19 in regulating the extent of splicing (27). If this were the case, viral proteins may compete with cellular proteins for sites on the viral RNA where splicing occurs. A change in the structural features of the RNA such as the presence or absence of internal methylations may alter the binding affinity of viral proteins relative to cellular proteins, which may prefer methylated sites on the RNA. The flow of RNA may therefore be shifted towards the production of more unspliced genome-length RNA. As an additional possibility, the presence of internal m^6A residues in helical regions of RNA is known to destabilize Watson-Crick base pairing (18) and therefore may alter the conformation of the RNA. This may also be of importance in determining the efficiency of splicing of viral RNA.

If internal m^6A residues are involved in the splicing of viral RNA, at least some of the methylations might be expected near the splice junctions. Mapping studies carried out to date have localized the methylations of the ASV genome RNA in the 3′ half of the RNA, which is consistent with such a location (5; K. Dimock, unpublished data). There was no evidence in these studies for the clustering of m^6A residues at splice junctions. However, the resolution used would probably not be sufficient to detect such clustering even if it did exist. Also, studies have not yet been done to compare the methylation sites of the viral RNAs present in infected cells with those in the genomic RNA. In fact, the only examples of mRNAs where the internal m^6As have been precisely located are in the late simian virus 40 16S and 19S mRNAs. It was found that, of the three m^6As per molecule, two were clustered at the 5′ end of the 16S mRNA, near but not at the sites of splicing (11).

We have shown that the major effects on viral mRNA metabolism and polypeptide synthesis occur at cycloleucine concentrations of >10 mM (Fig. 6 and 9). From the data given in Fig. 7 it can be seen that, at these cycloleucine concentrations, >80% of the internal m^6A methylations are inhibited. Since the genomic RNA of td B77 ASV contains approximately five m^6A residues per molecule (Fig. 6), it can be concluded that on the average four of these five m^6A residues are not absolutely required for the production of spliced mRNAs. Only when the AdoMet levels are lowered sufficiently to reduce methylation to less than an average of one m^6A per molecule is there a complete inhibition in the accumulation of spliced mRNA. It is possible that there may be selective inhibitions of some of the m^6A methylations (i.e., A-m^6A-C versus G-m^6A-C) and that certain sites of methylation, perhaps those which are critical for the splicing reaction, are more resistant to inhibition by the cycloleucine treatment. An example of selective inhibition has been shown for cap methylations in the presence of 40 mM cycloleucine; m^7G methylations proceed normally under these conditions, whereas ribose methylations of the penultimate guanosine residue are inhibited by 90% (16).

Our results suggest that the *env* mRNA is quite stable and that its half-life may be as long as 24 h. Under conditions in which we observed a complete inhibition in the accumulation of newly synthesized *env* mRNA, i.e., in the presence of 40 mM cycloleucine (Fig. 4), less than a twofold reduction in the steady-state levels of subgenomic RNA was obtained after a 24-h drug treatment (Table 2). Furthermore newly synthesized subgenomic *env* mRNA accumulates in td B77 ASV-infected cells at a relatively slow rate, and steady-state levels do not appear to be reached even after 24 h of labeling (Fig. 4). The genomic RNA pool in the infected cell appears to turn over more rapidly (half-life on the order of 8 h; C. M. Stoltzfus, K. Dimock, S. Horakami, and T. Ficht, manuscript in preparation). This might be expected since a substantial portion of this RNA is exported from the cell in virions, whereas little or none of the subgenomic RNA is packaged. Therefore, because of the stability of the *env* mRNA, relatively little of the genomic RNA would need to be spliced for sufficient amounts of *env* mRNA to accumulate in the infected cells.

ACKNOWLEDGMENTS

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